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# Antioxidant Capacity and Phenolic Content of Spinach As Affected by Genetics and Growing Season

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Total phenolic content and antioxidant capacity of 11 commercial cultivars and 15 advanced breeding lines of spinach were determined over two growing seasons known to vary in biotic and abiotic stresses. Flavonoid composition and content of fall-grown commercial cultivars and advanced breeding lines were also determined. Over-winter spinach, which was planted in late fall and harvested in the spring, had much higher levels of total phenolics and antioxidant capacity than spinach planted in early fall and harvested in late fall, indicating that growing conditions, as well as biotic and abiotic stresses, influenced phenolic metabolism. Genotype also appeared to play an important role in affecting phenolic metabolism and antioxidant capacity in spinach. Advanced breeding lines of spinach, which show increased disease resistance, had higher levels of total phenolics, individual and total flavonoids, and antioxidant capacity than commercial cultivars. Our results indicate that plant breeders can select for increased phenolic content to increase antioxidant capacity in spinach cultivars, or the crops can be grown in different seasons or under certain stress conditions to elevate levels of antioxidants.

**KEYWORDS: Spinach; genotype; phenolics; flavonoids; antioxidant capacity**

## INTRODUCTION

There is increasing evidence that fruits and vegetables may afford protection against numerous chronic diseases, including cancer, cardio- and cerebrovascular, ocular, and neurological diseases (*1*−*5*). The protective effect of fruits and vegetables has generally been attributed to their antioxidant constituents, including vitamins C and E, carotenoids, glutathione, flavonoids, and phenolic acids, as well as other unidentified compounds. Dietary antioxidant compounds are thought to protect against chronic diseases through mitigation of free radical damage to proteins, lipids, and DNA in humans. Because it is impractical to quantify all of the compounds in plants that exhibit antioxidant activity, assays have been developed to quantify total antioxidant capacity of plant extracts. Total antioxidant capacity of many fruits and vegetables has been determined by the oxygen radical absorbance capacity (ORAC) assay, which measures the ability of plant extracts to scavenge peroxyl radicals (*6*−*8*). These studies show that fruits and vegetables vary greatly in their total antioxidant capacity, and extracts with high levels of total phenolics typically have high ORAC values. Phenolic and ORAC levels in fruits and vegetables can be influenced by genetics, environmental growing conditions, maturation, and postharvest storage conditions. Although most work regarding these factors has been done on anthocyanin rich blueberries, blackberries, and strawberries (*9*−*14*), vegetables such as spinach, which also possess high ORACs (*8*), have received little attention.

Spinach (*Spinacia oleracea*) is an important dietary vegetable that is consumed fresh, in salad mixes, or after cooking in boiling water. The total flavonoid content of spinach is high (1000 mg/kg) (*15*), compared with those of other fruits and vegetables (*16*). Plant-derived polyphenolic flavonoids exhibit numerous biological and pharmacological properties (*16*−*18*) that could potentially afford protection against chronic diseases. Spinach flavonols, in particular, have been shown to possess antioxidant (*15*, *19*), antiinflammatory (*20*, *21*), antimutagenic (*22*), and anticarcinogenic (*23*) properties. The 10 predominant flavonoids in spinach include glucuronides and acylated di- and triglycosides of methylated and methylenedioxiderivatives of 6-oxygenated flavonols (*15*, *24*−*26*). Although the effect of storage and processing on flavonoid and antioxidant changes in fresh-cut spinach has been studied (*15*), no information is available concerning the effect of genetics and growing season on these important bioactive constituents.

This study was undertaken to determine how commercial cultivars (CC) and advanced breeding lines (ABL) of spinach vary in flavonoid content, and to determine how different growing seasons known to vary in biotic and abiotic stresses influence phenolic content and antioxidant capacity.

## MATERIALS AND METHODS

**Leaf Sampling.** Spinach leaves from 11 CC and 15 ABL were sampled from field-grown over-winter and fall spinach, which were

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cultivated at the University of Arkansas Vegetable Substation in Kibler, Arkansas. Over-winter spinach was planted on October 15, 1999 and harvested on March 21, 2000. Fall spinach was planted on September 1, 2000 and harvested on December 3, 2000.

At both harvest dates, 25 leaves from each CC and ABL were collected, placed in polyethylene bags in refrigerated coolers, and transported to the University of Arkansas Food Science Department within 2 h. Samples received at the Food Science Department were stored at −20 °C (for ∼2 months) until analyzed.

**Chemical Analyses.** *Chemicals.* R-phycoerythrin (R-PE), chlorogenic acid, and Folin−Ciocalteu reagent were purchased from Sigma Chemical Co. (St. Louis, MO). 6-Hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox) was obtained from Aldrich (Milwaukee, WI). AAPH was obtained from Wako Chemicals, USA, Inc. (Richmond, VA). HPLC-grade methanol and acetone were obtained from VWR (West Chester, PA).

*Sample Preparation for Determination of ORAC and Total Phenolics.* Frozen samples (500 g in triplicate) taken from each spinach source were homogenized for 2 min to the smallest possible particle size using a Black & Decker Handy Chopper Plus commercial blender. Pureed sub-samples were extracted (5 g/20 mL in triplicate) with ethanol/acetone/water/acetic acid (40:40:20:0.1). Samples were placed in screw-cap vials to prevent solvent evaporation and heated for 60 min in a 60 °C water bath. Samples were allowed to cool, then homogenized for 1 min using a Euro Turrax model T18 Tissuemizer (Tekmar-Dohrmann Corporation, Mason, OH). Extracts were filtered through Miracloth (CalBiochem, LaJolla, CA) and kept frozen (−20 °C) until analysis. Prior to ORAC and total phenolic assays, frozen extracts were thawed and diluted with phosphate buffer and deionized water, respectively.

*Sample Preparation for Determination of Flavonoids.* Flavonoids were extracted according to the method of Gil et al. (*15*). A 20-g fresh-weight sample was homogenized with 80 mL of MeOH/$H_2O$ (5:95) containing citric acid (0.5 g/L) and EDTA (0.5 g/L). Homogenate was filtered through Miracloth, and passed through a 0.45-$\mu$m-pore filter prior to HPLC analysis.

*ORAC Assay.* Oxygen radical absorbance capacity (ORAC) was measured using a modified version of Cao et al. (*6*) for use with a Perkin-Elmer HTSoft 7000 Plus Bio Assay Reader (Norwalk, CT). Concentration of reagents was identical to that of Cao and Prior (*6*) except for the working trolox standard (Aldrich) which was diluted to 10 $\mu$M prior to use in the assay. For the assay, 20 $\mu$L of each sample diluted 100-fold with phosphate buffer was mixed with 160 $\mu$L of $\beta$-phycoerythrin (3.73 mg/L, Sigma) in a clear 48-well Falcon microplate, and a baseline reading was obtained. As rapidly as possible, 20 $\mu$L of 320 mM 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH, Wako Chemicals, Richmond, VA) was added to each well using a multichannel pipet. The plate was agitated for 20 s prior to reading and for 5 s before each reading at 2-min intervals for 70 min. Excitation and emission filters were 535 and 560 nm, respectively. Data were expressed in micromoles of trolox equivalents per gram of fresh weight ($\mu$M TE/g).

*Total Phenolics Assay.* Total soluble phenolics in the ethanol/acetone/water/acetic acid extracts were determined with Folin−Ciocalteu reagent according to the method of Slinkard and Singleton (*27*) using chlorogenic acid as a standard. Results are expressed as chlorogenic acid equivalents per gram of fresh weight.

*Flavonoid Analysis.* Flavonoids were analyzed by HPLC as described by Gil et al. (*15*). Filtered extract (20 $\mu$L) was injected into a Waters Alliance model 2690 HPLC system coupled with an autosampler and model 996 photodiode array detector. Separations were achieved on a LiCrochart column (RP-18, 12.5 × 0.4 cm, 5-$\mu$m particle size; Merck, Darmstedt, Germany). Elution was performed using water/formic acid (19:1, v/v) (A) and HPLC-grade methanol (B) as the mobile phases, on a gradient starting with 10% B in A to reach 40% B at 30 min and 80% B at 40 min. The flow rate was 1 mL/min, and chromatograms were recorded at 280 and 350 nm. Peaks were quantified using external flavonoid standards that had been previously isolated from spinach and identified (*15*, *24*−*26*). Individual HPLC peaks were summed and expressed as total flavonoid content.

*Statistical Analyses.* Analysis of variance was performed using JMP software (*28*) to determine effects of growing season and cultivar on ORAC and phenolic content. Mean values were compared using Student's *t* test at 5% level. Correlation analysis between ORAC and phenolics was also performed using JMP software.

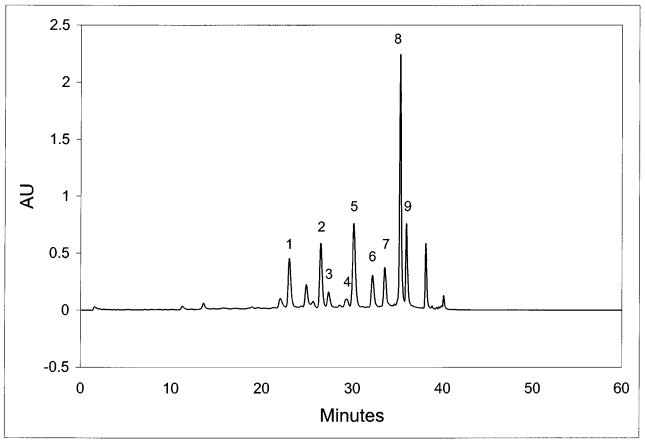


**Figure 1.** Typical HPLC chromatogram of spinach flavonoids. See **Table 2** for flavonoid identification.

## RESULTS

**Effect of Growing Season on ORAC and Total Phenolic Content of ABL and CC.** Total phenolic content and ORAC of CC and ABL varied greatly over the two growing seasons (**Table 1**). ORAC values ranged from 10.7 to 25.0 $\mu$M TE/g for all CC and ABL in the spring season, reflecting a 2.3-fold difference, whereas ORAC values ranged from 9.6 to 19.4 $\mu$M TE/g between CC and ABL in the fall season, reflecting a 2.0-fold difference. Advanced breeding lines had higher ORAC values than CC during both seasons ($P = 0.0025$). The ORAC mean for ABL in the spring season was 17.8 $\mu$M TE/g, compared with 16.1 $\mu$M TE/g for CC. In the fall season, the ORAC mean for ABL was 14.4 $\mu$M TE/g, compared with 12.3 $\mu$M TE/g for CC. ORAC values declined markedly from spring to fall seasons in 14 CC and ABL, remained relatively constant in 10 CC and ABL, and increased in 2 CC and ABL. The overall ORAC mean for the spring season was 17.1 $\mu$M TE/g, compared with 13.5 $\mu$M TE/g for the fall season ($P = 0.0001$), reflecting a 21% decrease over the two growing seasons.

Total phenolics ranged from 2291 to 4835 mg/kg for all CC and ABL grown in the spring season, reflecting a 2.1-fold difference, whereas total phenolics ranged from 1547 to 2408 mg/kg in the fall season, reflecting a 1.6-fold difference. Advanced breeding lines had higher levels of total phenolics than CC during both seasons ($P = 0.0504$). The total phenolic mean for ABL in the spring season was 3817.9 mg/kg, compared with 3387.8 mg/kg for CC. In the fall season, the total phenolic mean for ABL was 2019.2 mg/kg, compared with 1813.4 mg/kg for CC. Levels of total phenolics declined markedly from spring to fall seasons in all ABL and CC ($P = 0.0001$). The overall total phenolic mean for the spring season was 3633.6 mg/kg, compared with 1813.4 mg/kg for the fall season, reflecting a 50% decrease over the two growing seasons.

**Flavonoid Content of Spinach Cultivars and Breeding Lines Grown in Spring 2000.** Using extraction and HPLC conditions identical to those of Gil et al. (*15*), our chromatograms of spinach CC and ABL revealed similar profiles (**Figure 1**), with nine predominant flavonoids present that have previously been identified (*24*−*26*) (see **Table 2** for flavonoid identification). However, compound **4** was present in small quantities, or not detected in many samples, which prevented its quantification.

**Table 1.** Content of Phenolics and Oxygen Radical Absorbing Capacity (ORAC) of Spinach Cultivars Harvested in Spring and Fall 2000

| | spring 2000 | | fall 2000 | |
|---|---|---|---|---|
| cultivar/breeding line | ORAC[a] | phenolics[b] | ORAC | phenolics |
| | | Advanced Breeding Lines | | |
| 86-70 | 13.1 ± 0.1[c] | 3468.3 ± 16.9 | 13.4 ± 0.8 | 1989.6 ± 26.3* |
| 88-130 | 20.2 ± 0.2 | 4835.3 ± 97.0 | 13.4 ± 1.1* | 2032.8 ± 56.4* |
| 88-212 | 14.5 ± 0.3 | 2599.1 ± 61.4 | 12.2 ± 1.0 | 1822.3 ± 34.8* |
| 88-354 | 21.3 ± 0.3 | 4669.3 ± 29.5 | 19.4 ± 1.2 | 2408.4 ± 46.3* |
| 90-198 | 13.3 ± 0.1 | 3637.8 ± 14.9 | 18.0 ± 0.7* | 2290.6 ± 47.5* |
| 90-252 | 19.9 ± 0.2 | 3682.7 ± 40.0 | 15.9 ± 0.3* | 2100.1 ± 43.9* |
| 90-276 | 21.9 ± 0.3 | 4057.2 ± 36.0 | 11.8 ± 1.1* | 1842.8 ± 32.2* |
| 91-227 | 20.5 ± 0.2 | 3945.5 ± 55.7 | 17.6 ± 0.9* | 2193.7 ± 35.7* |
| 91-248 | 16.3 ± 0.2 | 3561.9 ± 6.9 | 17.3 ± 0.7 | 2246.9 ± 60.9* |
| 91-415 | 13.1 ± 0.3 | 3351.1 ± 25.9 | 13.5 ± 1.2 | 1755.8 ± 89.8* |
| 97-139 | 22.4 ± 0.1 | 4282.9 ± 50.3 | 12.4 ± 0.6* | 1704.0 ± 63.0* |
| 97-152 | 19.9 ± 0.5 | 3668.9 ± 59.6 | 17.4 ± 0.5* | 2286.9 ± 82.9* |
| 97-154 | 17.3 ± 0.2 | 4281.5 ± 20.1 | 11.3 ± 0.6* | 1947.1 ± 68.8* |
| 97-165 | 13.3 ± 0.2 | 3489.0 ± 5.0 | 9.9 ± 1.5 | 1715.1 ± 57.9* |
| 97-173 | 19.2 ± 0.1 | 3332.3 ± 27.9 | 12.4 ± 0.8* | 1951.7 ± 42.3* |
| means advanced breeding lines | 17.8 ± 0.2 | 3817.9 ± 36.4 | 14.4 ± 0.9* | 2019.2 ± 52.6* |
| | | Commercial Cultivars | | |
| Avon | 13.2 ± 0.3 | 2864.9 ± 26.0 | 10.8 ± 1.2 | 1988.9 ± 45.4* |
| Bolero | 13.4 ± 0.1 | 2291.8 ± 76.6 | 10.9 ± 1.5 | 1774.1 ± 89.7* |
| Coho | 12.2 ± 0.1 | 2904.6 ± 17.3 | 12.2 ± 0.4 | 1848.9 ± 90.9* |
| DMC 6609 | 20.4 ± 0.1 | 3388.7 ± 18.5 | 10.8 ± 0.4* | 1850.9 ± 68.1* |
| F380 | 25.0 ± 0.2 | 3832.6 ± 38.1 | 13.6 ± 0.8* | 2057.3 ± 36.0* |
| Fallgreen | 20.8 ± 0.4 | 3608.9 ± 32.6 | 12.6 ± 1.0* | 1547.4 ± 43.9* |
| Ozarka II | 14.4 ± 0.3 | 4544.4 ± 12.1 | 14.8 ± 0.1 | 1940.8 ± 105.8* |
| Samish | 10.7 ± 0.1 | 3609.6 ± 19.5 | 13.0 ± 0.1* | 1589.6 ± 69.2* |
| San Juan | 16.4 ± 0.2 | 3026.8 ± 27.8 | 14.2 ± 0.4* | 1908.6 ± 92.2* |
| St. Helens | 15.0 ± 0.3 | 3102.7 ± 8.5 | 12.8 ± 0.3* | 1811.4 ± 62.8* |
| Wintergreen | 15.8 ± 0.3 | 4090.9 ± 8.2 | 9.6 ± 1.0* | 1629.1 ± 34.2* |
| means commercial cultivars | 16.1 ± 0.2 | 3387.8 ± 25.9 | 12.3 ± 0.7* | 1813.4 ± 67.1* |
| overall means | 17.1 ± 0.2 | 3633.6 ± 32.0 | 13.5 ± 0.8* | 1932.1 ± 58.7* |

[a] Micromoles of Trolox equivalents per gram of fresh weight. [b] Milligrams of chlorogenic acid equivalents per gram of fresh weight. [c] Standard error of the mean ($N = 3$). * Indicates significant difference between growing seasons, Student's $t$ test ($P < 0.05$).

**Table 2.** Individual Flavonoids in Spinach Cultivars/Breeding Lines

| peak no. | flavonoid |
|---|---|
| 1 | Patuletin 3-*O*-$\beta$-D-glucopyranosyl(1→6)-[$\beta$-D-apiofuranosyl(1→2)]-$\beta$-D-glucopyranoside |
| 2 | Spinacetin 3-*O*-$\beta$-D-glucopyranosyl(1→6)-[$\beta$-D-apifuranosyl(1→2)]-$\beta$-D-glucopyranoside |
| 3 | Patuletin 3-*O*-$\beta$-D-(2″-feruloylglucopyranosyl)(1→6)-[$\beta$-D-apiofuranosyl(1→2)]-$\beta$-D-glucopyranoside |
| 4 | Spinacetin 3-*O*-$\beta$-D-(2″-*p*-coumaroylglucopyranosyl)(1→6)-[$\beta$-D-apiofuranosyl(1→2)]-$\beta$-D-glucopyranoside |
| 5 | Spinacetin 3-*O*-$\beta$-D-(2″-feruloylglucopyranosyl)(1→6)-[$\beta$-D-apiofuranosyl(1→2)]-$\beta$-D-glucopyranoside |
| 6 | Spinacetin 3-*O*-$\beta$-D-glucopyranosyl)(1→6)-$\beta$-D-glucopyranoside |
| 7 | Jaceidin 4′-glucuronide |
| 8 | 5,3′,4′-trihydroxy-3-methoxy-6:7-methylenedioxyflavone 4′-glucuronide |
| 9 | 5,4′-dihydroxy-3,3′-dimethoxy-6:7-methylenedioxyflavone 4′-glucuronide |

Individual flavonoids varied greatly between ABL and CC analyzed (**Table 3**): patuletin derivatives (compounds **1** and **3**) varied 4−5-fold; spinacetin derivatives (compounds **2**, **5**, and **6**) varied 4−17-fold; jaceidin (compound **7**) varied 4-fold; and flavone derivatives (compounds **8** and **9**) varied 2−5-fold among ABL and CC. Total flavonoids ranged from 807 to 2241 mg/kg, reflecting a 2.8-fold difference. ABL 97-152 contained appreciably higher levels of flavonoids than the other materials analyzed, whereas St. Helens, a CC, contained the lowest level. The CC Samish was unusual in that, in addition to containing the highest amount of total flavonoids among the CC analyzed, it contained no detectible amount of compound **8**, but contained the highest amount of compound **9**. In terms of composition, patuletin derivatives accounted for 21.8%, spinacetin derivatives were 32.4%, jaceidin was 6.3%, and flavones accounted for 39.5% of total flavonoids.

Similar to results obtained with total phenolics, ABL contained higher levels of flavonoid compounds **1**, **2**, **3**, **5**, **7**, and **8** than CC ($P < 0.05$ for all compounds). The mean total flavonoid content of ABL, 1267 mg/kg, was significantly greater than the mean of CC, 1099 mg/kg ($P = 0.0010$). The mean total flavonoid content of all ABL and CC, 1199 mg/kg, was slightly higher than the concentration of 1036 mg/kg reported by Gil et al. (*15*) for an unknown spinach cultivar.

**Relationships between Phenolics and ORAC.** A significant linear relationship was observed between total phenolics and ORAC in both spring ($r_{xy} = 0.50$) and fall ($r_{xy} = 0.72$) seasons. The relationship between total phenolics and ORAC over both growing seasons was also significant ($r_{xy} = 0.63$). For spinach grown in the spring season, a significant linear relationship was observed between total flavonoids and ORAC ($r_{xy} = 0.71$). Two individual flavonoid compounds, **1** and **3**, also correlated well with ORAC ($r_{xy} = 0.74$ and $r_{xy} = 0.71$, respectively).

## DISCUSSION

**Effect of Growing Season on ORAC and Total Phenolic content of ABL and CC.** Higher levels of total phenolics and

**Table 3.** Individual Flavonoids of Spinach Cultivars Harvested in Spring 2000[a]

| cultivar/ breeding line | 1 | 2 | 3[b] | 5 | 6[c] | 7 | 8 | 9[d] | total |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Advanced Breeding Lines | | | | | |
| 86-70 | 219.9 ± 14.8[e] | 94.4 ± 4.9 | 130.8 ± 12.6 | 127.2 ± 7.4 | 96.9 ± 6.7 | 57.6 ± 2.1 | 185.4 ± 13.7 | 273.5 ± 13.7 | 1165.9 ± 63.8 |
| 88-130 | 168.1 ± 2.6 | 103.4 ± 1.2 | 102.3 ± 4.5 | 125.0 ± 1.6 | 52.9 ± 0.7 | 36.5 ± 0.6 | 163.9 ± 1.2 | 315.9 ± 5.4 | 1068.0 ± 8.2 |
| 88-212 | 121.6 ± 18.9 | 86.8 ± 8.8 | 68.8 ± 13.3 | 96.6 ± 11.3 | 54.7 ± 6.6 | 30.5 ± 3.4 | 154.2 ± 14.4 | 333.3 ± 19.9 | 946.5 ± 96.3 |
| 88-354 | 288.1 ± 9.1 | 99.5 ± 2.8 | 105.8 ± 3.1 | 157.9 ± 5.4 | 112.4 ± 5.6 | 54.3 ± 2.0 | 177.7 ± 7.9 | 300.1 ± 9.2 | 1295.8 ± 35.7 |
| 90-198 | 233.5 ± 15.2 | 123.9 ± 7.9 | 118.4 ± 6.6 | 134.8 ± 10.1 | 102.0 ± 16.0 | 38.6 ± 4.4 | 149.4 ± 20.4 | 468.6 ± 6.5 | 1369.2 ± 87.3 |
| 90-252 | 271.7 ± 8.9 | 114.6 ± 3.5 | 126.1 ± 5.9 | 132.4 ± 6.4 | 101.2 ± 7.9 | 35.9 ± 1.4 | 199.9 ± 4.2 | 279.8 ± 4.0 | 1261.7 ± 30.9 |
| 90-276 | 133.9 ± 10.1 | 89.5 ± 3.9 | 93.4 ± 12.3 | 121.1 ± 5.7 | 99.2 ± 8.0 | 39.2 ± 1.9 | 130.2 ± 8.4 | 411.8 ± 4.7 | 1118.4 ± 48.9 |
| 91-227 | 341.6 ± 13.1 | 149.6 ± 6.9 | 197.2 ± 4.3 | 171.9 ± 6.7 | 107.0 ± 8.0 | 46.8 ± 1.8 | 223.1 ± 10.3 | 481.3 ± 6.5 | 1718.5 ± 57.0 |
| 91-248 | 303.9 ± 25.7 | 218.9 ± 22.9 | 125.9 ± 10.0 | 197.9 ± 19.8 | 58.5 ± 13.9 | 49.7 ± 5.6 | 146.4 ± 30.6 | 483.5 ± 35.7 | 1584.8 ± 164.6 |
| 91-415 | 136.4 ± 14.5 | 74.8 ± 5.1 | 72.8 ± 6.4 | 71.6 ± 6.4 | 37.3 ± 6.2 | 18.1 ± 1.9 | 111.8 ± 11.7 | 414.4 ± 19.6 | 937.2 ± 70.4 |
| 97-139 | 105.4 ± 17.7 | 74.7 ± 1.9 | 58.0 ± 14.6 | 68.7 ± 4.5 | 37.3 ± 4.9 | 22.1 ± 0.9 | 116.4 ± 12.1 | 358.2 ± 15.6 | 827.5 ± 68.2 |
| 97-152 | 449.6 ± 18.5 | 206.9 ± 6.6 | 201.9 ± 1.4 | 264.7 ± 8.1 | 103.9 ± 10.5 | 73.8 ± 3.2 | 221.9 ± 14.6 | 718.2 ± 28.9 | 2241.1 ± 33.1 |
| 97-154 | 203.2 ± 14.8 | 123.8 ± 4.4 | 65.7 ± 4.2 | 145.4 ± 6.5 | 44.0 ± 2.1 | 40.1 ± 0.9 | 120.9 ± 5.3 | 381.4 ± 23.7 | 1124.6 ± 59.8 |
| 97-165 | 174.1 ± 28.6 | 103.1 ± 12.3 | 110.4 ± 28.1 | 114.8 ± 15.4 | 74.2 ± 8.6 | 35.3 ± 4.2 | 189.5 ± 28.7 | 372.8 ± 46.1 | 1174.0 ± 171.9 |
| 97-173 | 190.8 ± 17.4 | 108.9 ± 9.2 | 99.5 ± 9.5 | 120.7 ± 8.2 | 59.2 ± 8.4 | 37.8 ± 3.3 | 151.2 ± 18.8 | 369.4 ± 27.8 | 1137.5 ± 100.7 |
| means advanced breeding lines | 24.6 ± 14.9* | 117.0 ± 6.5* | 111.9 ± 7.1* | 137.5 ± 7.7* | 76.8 ± 4.6 | 40.1 ± 2.1* | 164.0 ± 6.3* | 395.2 ± 18.1 | 1267.2 ± 57.6* |
| | | | | Commercial Cultivars | | | | | |
| Avon | 168.4 ± 10.1 | 118.1 ± 2.2 | 49.6 ± 5.1 | 123.3 ± 1.6 | 99.9 ± 8.1 | 48.5 ± 2.8 | 137.1 ± 8.5 | 477.4 ± 30.4 | 1222.3 ± 47.8 |
| Bolero | 103.1 ± 8.3 | 53.6 ± 4.5 | 42.4 ± 11.3 | 72.3 ± 8.3 | 76.8 ± 9.8 | 30.3 ± 3.3 | 155.9 ± 16.2 | 388.1 ± 23.7 | 922.4 ± 74.1 |
| Coho | 171.8 ± 14.0 | 111.5 ± 5.0 | 52.6 ± 16.6 | 139.0 ± 6.4 | 48.7 ± 4.0 | 37.6 ± 2.1 | 121.7 ± 8.8 | 442.9 ± 27.2 | 1126.0 ± 114.1 |
| DMC 6609 | 197.7 ± 14.6 | 65.2 ± 3.5 | 91.4 ± 0.6 | 83.6 ± 7.1 | 217.5 ± 9.5 | 42.8 ± 1.1 | 84.8 ± 2.3 | 201.5 ± 27.8 | 984.6 ± 12.9 |
| F 380 | 239.4 ± 18.7 | 105.8 ± 6.8 | 105.4 ± 15.8 | 114.3 ± 5.6 | 101.8 ± 16.3 | 34.9 ± 1.5 | 176.3 ± 22.6 | 211.5 ± 2.7 | 1089.4 ± 84.7 |
| Fallgreen | 144.8 ± 14.4 | 75.7 ± 5.5 | 102.2 ± 11.3 | 84.9 ± 5.7 | 65.0 ± 5.5 | 27.8 ± 2.1 | 119.6 ± 11.5 | 399.7 ± 15.3 | 1019.7 ± 68.2 |
| Ozarka II | 194.6 ± 19.3 | 104.0 ± 2.6 | 133.1 ± 20.2 | 117.3 ± 6.6 | 61.0 ± 2.5 | 32.8 ± 1.5 | 168.6 ± 8.1 | 479.1 ± 28.1 | 1290.5 ± 88.9 |
| Samish | 213.2 ± 19.1 | 117.2 ± 1.5 | 101.1 ± 6.3 | 111.6 ± 3.9 | 17.6 ± 7.9 | 28.2 ± 2.6 | ND | 1131.3 ± 12.0 | 1720.1 ± 8.2 |
| San Juan | 307.9 ± 21.1 | 145.4 ± 1.7 | 121.4 ± 4.6 | 166.1 ± 7.9 | 52.0 ± 2.1 | 37.1 ± 1.4 | 161.8 ± 1.4 | 348.0 ± 1.8 | 1339.8 ± 38.0 |
| St. Helens | 107.7 ± 3.5 | 76.7 ± 2.3 | 63.6 ± 2.5 | 91.3 ± 2.7 | 15.6 ± 1.8 | 27.1 ± 1.1 | 101.3 ± 10.3 | 323.6 ± 11.4 | 806.8 ± 33.2 |
| Wintergreen | 109.9 ± 9.0 | 99.1 ± 5.9 | 40.4 ± 6.3 | 133.9 ± 9.1 | 44.3 ± 2.9 | 33.3 ± 1.7 | 139.9 ± 3.9 | 227.9 ± 8.9 | 878.8 ± 43.4 |
| means commercial cultivars | 168.7 ± 11.5 | 99.5 ± 5.9 | 77.0 ± 6.6 | 111.4 ± 5.3 | 69.7 ± 9.6 | 34.5 ± 1.4 | 125.3 ± 8.7 | 416.8 ± 42.0 | 1099.2 ± 48.2 |
| overall means | 201.9 ± 10.5 | 109.9 ± 4.6 | 97.8 ± 5.4 | 126.9 ± 5.3 | 73.9 ± 4.7 | 37.8 ± 1.4 | 148.3 ± 5.6 | 403.9 ± 20.0 | 1199.0 ± 40.4 |

[a] See **Table 2** for flavonoid identification. Values are milligrams of each flavonol per kilogram of fresh weight. [b] Data quantified as peak 3. [c] Data quantified as peak 5. [d] Data quantified as peak 8. [e] Standard error of the mean ($N = 3$). * Indicates significant difference between advanced breeding lines and commercial cultivars, Student's *t* test ($P < 0.05$).

antioxidant capacity in spring- versus fall-grown spinach may be attributed to differences in environmental growing conditions and pathological and physiological stresses incurred over the two growing seasons. Spinach grown over winter and harvested in early spring in the mid-south region of the U. S. incurs higher growing temperatures and greater light intensity, is challenged more by disease, and is more susceptible to bolting than spinach grown and harvested in the fall. Many of these biotic and abiotic stresses are known to induce phenylpropanoid metabolism in plants (*29, 30*). Commercial cultivars and ABL of spinach that synthesize higher levels of phenolics in response to biotic and abiotic stresses could potentially have greater resistance to common spinach diseases.

Interestingly, ORAC values were 27% lower in fall-grown than in spring-grown spinach in all ABL and CC, whereas levels of total phenolics were 88% lower. This discrepancy may be attributed to greater synthesis of glycosylated and acylated flavonoids or additional phenolics in spring-grown spinach, which possess little to no antioxidant capacity (*15*). The large differences observed in ORAC and total phenolics between the ABL and CC over the two growing seasons show that it is important for plant breeders to consider environmental effects when selecting spinach cultivars for enhanced antioxidant capacity.

The wide range of ORAC values (9.6−25.0 $\mu$M TE/g FW) observed among spinach cultivars over the two growing seasons was generally higher than the previously reported value of 12.6 uM TE/g FW for an unknown spinach cultivar (*8*). The higher ORAC values obtained in our study may be attributed to differences in extraction protocol, genetics, and environmental growing conditions. Our data confirm that spinach has a high antioxidant capacity, and that considerable variation in antioxidant capacity exists among spinach cultivars.

Advanced breeding lines contained higher levels of total phenolics and antioxidant capacity then CC. Advanced selections from the breeding program have undergone repeated field selections for disease resistance, and have shown polygenic resistance to white rust and races 3 and 4 of downy mildew (*31*). One ABL (88−354) and several CC (Wintergreen, F380, Fallgreen, and Ozarka II), developed in the breeding program for disease resistance, synthesized high levels of phenolics, resulting in enhanced ORAC when challenged by stressful growing conditions in the spring. Thus, it appears that genetics may play a large role in the ability of spinach cultivars to respond to biotic and abiotic stresses, which ultimately impacts their phenolic content and antioxidant capacity. Studies are underway to determine if the stress-induced phenylpropanoids confer disease protection through phytoalexic activity.

**Flavonoid Content of Spinach Cultivars and Breeding Lines Grown in Spring 2000.** Spinach is unique in that it does not contain flavonoids common to other fruits and vegetables, such as flavonols (quercetin, myrecetin, and kaemperol) and flavones (apigenin, luteolin) (*32*). The flavonoids unique to spinach include glucuronides and acylated di- and triglycosides of methylated and methylenedioxiderivatives of 6-oxygenated flavonols (*15*, *24*−*26*). In our study we quantified eight of the

ten flavonoids previously reported, but we were unable to quantify spinacetin 3-*O*-*β*-D-(2″-*p*-coumaroylglucopyranosyl)-(1→6)-[ *β*-D-apiofuranosyl (1→2)]-*β*-D-glucopyranoside or spinacetin 3-*O*-*β*-D-(2″-feruloylglucopyranosyl)) (1→6))- *β*-D-glucopyranoside, which were present in low amounts, or not detected, in many of the ABL and CC. The levels of individual flavonoids were strongly affected by genotype, indicating that spinach ABL and CC vary in their capacity to synthesize flavonoids. Consistent with total phenolic results, ABL of spinach had higher levels of many individual flavonoids than CC, indicating that selection for disease resistance has resulted in flavonoid-enriched germplasm. Several of the ABL (97-152, 91-227, and 91-248) contained high levels of flavonoids, whereas others (97-139, 91-415, and 88-212) contained low levels. Among CC, Samish and San Juan contained high levels of flavonoids, and St. Helens and Wintergreen contained low levels. Samish was unusual in that it contained no detectible amount of compound **8** (5,3′, 4′-trihydroxy-3-methoxy-6,7-methylendioxyflavone 4′-glucuronide), but contained the highest amount of compound **9** (5,4′-dihydroxy-3,3′-dimetoxy-6,7 methylenedioxyflavone 4′-glucuronide), suggesting that enzymes affecting hydroxylation or dehydroxylation reactions are genetically altered. Because ABL and CC with elevated flavonoid content typically exhibit increased antioxidant capacity, plant breeders should continue to select for increased levels of total phenolics, as long as flavor attributes are not adversely affected.

**Relationships between Phenolics and ORAC.** A positive linear relationship between total phenolics and antioxidant capacity was observed in spinach. However, the relationship between total phenolics and ORAC in spinach was less pronounced than the highly linear relationship observed between total phenolics and ORAC in blueberries, blackberries, raspberries, and strawberries (*9*, *11*). The lower correlation observed between these variables could be explained by the complex flavonoid composition of spinach. Only two of the flavonoid compounds (**1** and **3**) in spinach showed significant free radical scavenging activity against the 2,2-diphenyl-1-picrylhydrazyl radical (*15*). Both of the patuletin derivatives have a 3′,4′-dihydroxyl grouping, an important structural feature known to increase the free-radical scavenging capacity of flavonoids (*18*). Interestingly, the two patuletin derivatives, compounds **1** and **3**, correlated highly with ORAC in our study ($r_{xy} = 0.74$ and 0.71, respectively), which indirectly corroborates their contribution to antioxidant capacity. Our results concerning the relationship between total phenolics, flavonoids, and ORAC are also consistent with those of Guo et al. (*33*), who found that electroactive components in spinach extracts determined by HPLC coupled with coulometric array detection did not correlate with ORAC as well as electroactive components measured in other fruits and vegetables. Thus, it appears that although spinach is a rich source of flavonoids, only compounds with a 3′,4′-dihydroxyl grouping, which accounted for only 22% of the total flavonoid content, show significant free radical scavenging capacity. Besides flavonoids, other water-soluble compounds not measured in the study could also contribute significantly to ORAC. Isomers of *p*-coumaric acid and uridine extracted from spinach leaves are reported to be potent inhibitors of linoleic acid autoxidation (*34*).

In conclusion, over-winter spinach, which is harvested in the spring, had much higher levels of total phenolics and antioxidant capacity than spinach grown in the fall, indicating that environmental growing conditions as well as biotic and abiotic stresses influenced phenylpropanoid metabolism. Genotype also appeared to play an important role in affecting phenolic metabolism and antioxidant capacity in spinach. Advanced breeding lines of spinach with increased disease resistance had higher levels of total phenolics, individual flavonoids, and antioxidant capacity than CC. Our results indicate that plant breeders can select for increased phenolic content in spinach cultivars to increase antioxidant capacity, and possibly confer greater disease resistance.

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